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The Role of Oncogenes in Chemical Carcinogenesis

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Proto-oncogenes are cellular genes that are expressed during normal growth and developmental processes. Altered versions of normal proto-oncogenes have been implicated in the development of human neoplasia. In this report, we show the detection of activated proto-oncogenes in various spontaneous and chemically induced rodent tumors. The majority of activated proto-oncogenes found in these tumors are members of the *ras* gene family and have been activated by a point mutation. Characterization of the activating mutation may be useful in determining whether this proto-oncogene was activated by direct interaction of the chemical with the DNA. Comparison of activating lesions in spontaneous versus chemically induced tumors should be helpful in determining whether the chemical acts via a genotoxic or a nongenotoxic mechanism. All of this information may be helpful in the assessment of potential carcinogenic hazards of human exposure to chemicals.

Introduction

Recent evidence suggests that neoplastic development, at least in part, is the result of the abnormal activation of a small set of cellular genes. These genes, termed proto-oncogenes, were originally discovered as the transduced genes of acute transforming retroviruses (*1–3*). Subsequent studies have established that these proto-oncogenes can also be activated as oncogenes by mechanisms independent of retroviruses (*4*). Mechanisms for the conversion of proto-oncogenes to activated oncogenes include point mutations, gene amplification, chromosomal rearrangements, and promoter insertion (Fig. 1). The activation of proto-oncogenes by genetic alterations results in altered levels of expression of the normal protein product, or in normal or altered levels of expression of an abnormal protein.

Proto-oncogenes are expressed during regulated growth such as embryogenesis, regeneration of damaged liver, and stimulation of cell mitosis by growth factors. Proto-oncogenes are highly conserved. They are detected in species as divergent as yeast, Drosophila, and humans. Proto-oncogenes include genes that encode for growth factors (*sis*), growth factor receptors (*neu, erbB, fms*), regulatory proteins in signal transduction (*ras* family), nuclear regulatory proteins (*myc, myb, fos*), and tyrosine kinases (*scr, abl, ras*). Thus, the encoded proteins appear to play a crucial role in normal cellular growth and/or differentiation.

The activation of proto-oncogenes in spontaneous and chemically induced tumors has been extensively studied during the past several years. Although oncogenes such as *ras* and *myc* can complement each other in the malignant transformation of a cell *in vitro* (*2*), the number of proto-oncogenes that must be activated in the multistep process of carcinogenesis is unclear at present. Also, new evidence from several laboratories suggests that in addition to the activation of positive factors (oncogenes), the loss of negative regulatory functions (tumor suppressor genes) may also be a necessary but distinct step in neoplastic development (*5*). This paper will discuss the detection of activated oncogenes in rodent tumors and the implication of oncogenes in risk analysis of carcinogen-induced rodent tumor data.

Detection of Activated Oncogenes in Tumors

Detection of activated oncogenes in neoplasia can be achieved by using several different techniques depending on how the particular oncogene might be activated. Abnormal expression of oncogenes in tumors due to amplification of the gene may be detected by dot blot or Southern blot analysis. Increased expression due to deregulation of the gene may be detected by dot blot as well as Northern blot analysis. Chromosomal translocations may be detected by cytogenetic analysis. Ex-

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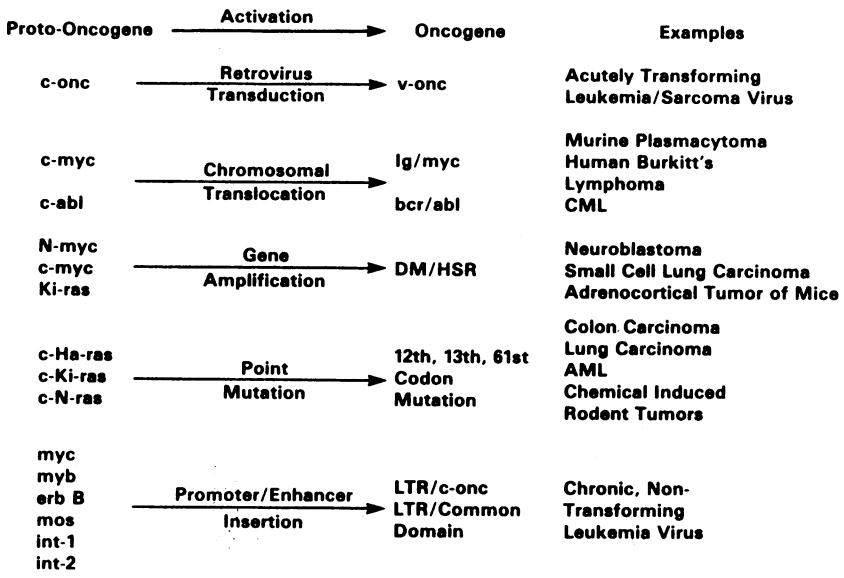

FIGURE 1. Mechanisms of proto-oncogene activation.

amples of abnormal expression of oncogenes detected in human tumors and tumor cell lines are shown in Table 1.

A number of oncogenes present in human tumors as well as animal tumors have been detected by the NIH/3T3 transfection assay. The NIH/3T3 transfection technique involves the ability of the NIH/3T3 mouse fibroblast to accept and express genes from donor tumor DNA, resulting in the formation of transformed cells. Only a few years ago, Shih et al. (*6*) were the first to show that DNA from carcinogen-transformed cell lines could cause transformation of NIH/3T3 cells after transfection. This transformation was characterized by a change in the morphology of the NIH/3T3 cells and by anchorage-independent growth. Other investigators using this technique were then able to show that dominant transforming genes or oncogenes were present in human tumors and in carcinogen-induced animal tumors. An extension of the NIH/3T3 transfection assay that affords greater sensitivity is the nude mouse tumorigenicity assay (*7*). This involves cotransfection of NIH/3T3 cells with tumor DNA and a selectable marker gene. The selected cells are then injected SC into the immunocompromised mice. The tumors that develop from the nude mice are then analyzed using the techniques described earlier to characterize the activated oncogenes.

Table 1. Abnormal expression of oncogenes in human tumors and tumor cell lines.

Mechanism	Tumor type	Oncogene	Reference
Amplification	Breast tumor	*neu*	(*24*)
Amplification	Squamous cell carcinoma	c-*erbB*	(*25*)
Amplification	Small cell lung carcinoma	c-*myc*	(*26*)
Amplification	Small cell lung carcinoma	L-*myc*	(*27*)
Amplification	Neuroblastoma	N-*myc*	(*28*)
Amplification	Acute myelogenous leukemia	c-*myb*	(*29*)
Translocation	Chronic myelogenous leukemia	c-*abl*	(*30*)
Translocation	Burkitt's lymphoma	c-*myc*	(*31*)

The majority of activated genes in human tumors detected by the NIH/3T3 assay have been members of the *ras* gene family: the H-*ras*, the K-*ras*, and the N-*ras*. Early studies using the NIH/3T3 assay showed only *ras* gene activation in a low percentage of the human tumors (approximately 10%). Later studies have shown that other oncogenes can also be detected in the human tumors by this assay, including the *lca* (*8*), *hst* (*9*), and the *trk* (*10*) oncogenes. In addition, the percentage of certain tumor types that test positive for activated *ras* oncogenes are higher than 10%. For example, Verlaan-deVries et al. (*11*) detected activated *ras* genes in the 27% of acute myeloid leukemia examined. Ananthaswamy et al. (*12*) detected Ha-*ras* genes in four of six human squamous cell carcinomas examined. The addition of the tumorigenicity parameter to the assay system appears to improve the efficiency in detection of activated oncogenes in human tumors.

A variety of animal tumor model systems have also been examined for activated genes using the NIH/3T3 assay. These include spontaneous tumors in rats and mice, tumors that arise after single or multiple doses of carcinogen, and tumors that arise after long-term exposure to a carcinogen. Examples of the activated genes in the different tumor model systems are shown in Table 2. Like the human tumors, the majority of activated oncogenes detected in the animal tumors are members of the *ras* gene family. Other oncogenes have also been detected in animal tumors using the NIH/3T3 assay (Table 3). One example is the activated *neu* oncogene found in nervous tissue tumors induced in rats by transplacental exposure to *N*-methyl-*N*-nitrosourea (MNU) or *N*-ethyl-*N*-nitrosourea (ENU). The c-*raf* on-

Table 2. Activated oncogenes in rodent tumor models.

Model	Tumor	Number positive/ Number tested	Oncogene	Reference
Spontaneous	Mouse liver	17/27	H-*ras* (15)[a], *raf* (1), unknown (1)	(*18,32*)
Single dose	Rat	1/37	H-*ras* (1)	(*18*)
NMU	Rat mammary	61/71	H-*ras* (61)	(*14*)
DMBA	Rat mammary	6/29	H-*ras* (6)	(*15*)
Single dose, neonatal				
HO-AAF[b]	Mouse liver	10/10	H-*ras* (10)	(*33*)
HO-DHE	Mouse liver	11/11	H-*ras* (10), K-*ras* (1)	(*33*)
VC	Mouse liver	10/10	H-*ras* (10)	(*33*)
DEN	Mouse liver	14/33	H-*ras* (14)	c
Multiple doses				
DMN-OME	Rat renal	10/35	K-*ras*	(*34*)
Aflatoxin B_1	Rat liver	10/11	K-*ras* (2), unknown (9)	(*35*)
Continuous dose				
TNM	Rat and mouse liver	18/19, 10/10	K-*ras* (18), K-*ras* (10)	(*19*)
Furan	Mouse liver	13/29	H-*ras* (10), *raf* (1), K-*ras* (2)	(*23*)
Furfural	Mouse liver	13/16	H-*ras* (9), K-*ras* (1), unknown (3)	(*23*)
Benzidine-derived dyes	Rat[d]	34/58	H-*ras* (31), N-*ras* (3)	e
DEN	Rat liver	1/12	Unknown	c
Initiation-promotion				
DEN-Farber protocol	Rat liver	0/20	—	(*36*)
DEN + PB	Rat liver	0/10	—	c
DEN + EE_2	Rat liver	2/19	non-*ras* (2)	f
DMBA + TPA	Mouse skin	33/37	H-*ras*	(*37*)
Transplacental dose				
ENU	Rat neuroblastomas	3/3	*neu* (3)	(*38*)
MNU	Rat schwannomas	10/13	*neu* (10)	(*16*)

[a] Numbers in parentheses are the number of positive samples with that oncogene.
[b] Abbreviations: HO-AAF, *N*-hydroxy-2-acetylaminofluorene; HO-DHE, 1'-hydroxy-2',3'-dehydroestragole; VC, vinyl carbamate; DEN, *N*-nitrosodiethylamine; DMN-OMe, methyl(methoxymethyl)nitrosamine; PB, phenobarbital; EE_2, ethinyl estradiol. For other abbreviations, see text.
[c] Stowers et al., unpublished data.
[d] Benzidine-derived dye-induced rat tumors include preputial gland tumors, squamous cell carcinomas, basal cell tumors, clitoral gland tumors, and mammary tumors.
[e] Reynolds and Anderson, unpublished data.
[f] Goodrow et al., unpublished data.

cogene has also been detected in mouse liver tumors. The detection of unusual mutations in the *ras* genes, as well as the identification of new classes of oncogenes in animal tumors, should be enhanced by the addition of the nude mouse tumorigenicity assay.

Activation of Oncogenes by Carcinogens

Studies in animal tumor model systems suggest that the chemicals or radiation may play a role in the activation of oncogenes by point mutation. Point mutations resulting in the activation of *ras* proto-oncogenes in several chemically induced rodent tumors have been consistent with the known alkylation patterns of the carcinogens (*13*). For example, the mutation at the 12th codon of the H-*ras* gene detected in rat mammary tumors induced by methylnitrosourea (*14*) is consistent with the formation of the 0^6 methylguanine adduct. The activating mutation in the 61st codon of the H-*ras* gene found in 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary tumors and skin tumors is consistent with DMBA binding to adenosine residues (*15*). The point mutation that activates the *neu* proto-oncogene in peripheral nervous system tumors in rats induced by ENU or MNU probably results from the binding of these potent genotoxic chemicals to DNA (*16*). "Hot spots" for activating mutations in these oncogenes have been observed (*17*). For example, the GGA→GAA mutation observed in the 12th codon of *ras* oncogenes detected in MNU-induced mammary carcinomas (*14*) is always at the second guanine of this codon, even though a similar mutation at the first guanine could also produce an activated *ras* oncogene. If the sequence of specificity for the binding of carcinogens to DNA corresponds to a known biological hot spot in an oncogene, then this chemical can be a very potent carcinogen.

Several studies have shown that oncogene activation may not be the direct result of chemical interaction with DNA. Activated oncogenes have been detected in a number of different types of tumors in the mouse. This

Table 3. Non-*ras* oncogenes detected in human and rodent tumors.

Tumor	Treatment	Oncogene	Reference
Neuroblastomas (R)[a]	ENU	*neu*	(*38*)
Schwannomas (R)	MNU	*neu*	(*16*)
Stomach carcinomas (H)	—	*hst*	(*9*)
Colon carcinomas (H)	—	*trk*	(*10*)
Hepatocellular carcinomas (M)	Untreated	*raf*	(*23*)
Hepatocellular carcinoma (H)	—	*lca*	(*8*)
Hepatocellular carcinomas (R)	AFB_1[b]	?	(*35*)
Hepatocellular carcinoma (M)	Untreated	?	(*23*)
Hepatocellular carcinomas (M)	Furfural	?	(*23*)
Pulmonary adenocarcinoma (M)	Untreated	?	c
Nasal squamous carcinomas (R)	MMS	?	(*13*)
Skin carcinoma (M)	DMBA	?	(*17*)
Skin carcinoma (M)	DB[c,h]ACR	?	(*17*)

[a] Letters in parentheses indicate species in which tumors occur. R, rat; M, mouse; H, human.

[b] Abbreviations: AFB_1, aflatoxin B_1; MMS, methylmethanesulfonate; DB[c,h]ACR, dibenz[c,h]acridine

[c] U. Candrian and M.W. Anderson, unpublished data.

Table 4. Detection of activated K-*ras* oncogene in tetranitromethane-induced lung tumors in mice and rats.

DNA source	Transforming K-*ras* gene with GGT→GAT mutation in 12th codon,[a] number positive/number tested
Rat adenocarcinoma	12/12 (100%)
Rat squamous cell carcinoma	3/4 (75%)
Rat adenosquamous carcinoma	3/3 (100%)
Mouse adenocarcinoma	8/8 (100%)
Mouse adenoma	2/2 (100%)

[a] The presence of transforming genes was detected by NIH/3T3 transfection and/or oligonucleotide hybridization, and the frequency of transforming genes is represented by the numbers in parentheses.

implies that activation of oncogenes in long-term carcinogenic studies in the mouse may or may not be the result of direct interaction of the chemical with DNA (U. Candrian, unpublished data; *18*). It is possible in some instances that the chemical did activate the oncogene directly and is consistent with the chemical binding to the DNA. In other instances, the chemical may have increased the background tumor incidence by a mechanism such as cytotoxicity or receptor-mediated promotion. If the pattern of activated oncogenes in the chemically induced tumors is different from that in the spontaneously occurring tumors, then the chemical probably caused the mutations, at least in some of the tumors. One example in which the chemical's role in oncogene activation is not known is the activated K-*ras* oncogene detected in tetranitromethane (TNM)-induced rat and mice lung tumors. TNM is a mutagen and an irritant. However, the interactions between TNM and DNA are not known. In a recent long-term carcinogenesis study conducted by the NTP, chronic exposure to TNM resulted in a high incidence of primary lung tumors in Fischer 344 rats and B6C3F1 mice (*19*). K-*ras* oncogenes with a GGT→GAT mutation in the 12th codon were observed in 18 of 19 rat lung tumors and 10 of 10 mouse lung tumors (Table 4). The activation of the K-*ras* oncogene in these TNM-induced lung tumors may be the result of one or more actions of the chemical: a direct consequence of TNM-induced DNA damage; the tumors may be spontaneously occurring; enhancement of spontaneously occurring K-*ras* by TNM-induced cell replication; or combination of direct TNM-induced DNA damage and enhancement of spontaneous occurrence.

It is a distinct possibility that these activated K-*ras* oncogenes with GC→AT transitions in the second base of the 12th codon are spontaneous, since an activated K-*ras* with the same mutation was observed in a spontaneously occurring pulmonary adenocarcinoma in the B6C3F1 mouse (U.Candrian, unpublished data). Even though spontaneous lung tumors in the Fischer 344 rat were not observed in this study, it is still possible that the irritant property of TNM could have promoted the cells, which activated the K-*ras* or enhanced the spontaneously occurring K-*ras*. The reproducible detection of the K-*ras* in lung tumors of mice and rats suggests that TNM could have directly induced the mutation. In support of this conclusion, mutagenicity studies have shown that TNM causes mutant bacterial strains to revert to the wild type by the same GC→AT transition. Studies on the possible interactions of TNM with DNA are required to precisely determine the origin of the activated K-*ras* oncogenes in these TNM-induced lung tumors.

Although gene amplification and chromosomal translocation have been observed in several types of human tumors, these activating mechanisms have not been extensively observed or studied in spontaneous or chemically induced rodent tumors. Sawey et al. (*20*) did observe c-*myc* gene amplification and restriction polymorphisms in addition to activated K-*ras* genes in rat skin tumors induced by ionizing radiation. Quintanilla et al. (*21*) suggested that amplification of the mutated H-*ras* gene may be involved in the progression of mouse skin papillomas to carcinomas. Further studies are required to determine the possible role of chemicals and radiation in the activation of proto-oncogenes by gene amplification, chromosomal translocation, and other mechanisms that can alter gene expression.

Carcinogen-induced rodent tumor models may be useful in determining the temporal activation of oncogenes in tumor development. Evidence in several animal studies suggests that activation of the *ras* proto-oncogene is an early event. The activated *ras* gene has been detected in many benign tumors, including mouse skin papillomas, mouse lung and liver adenomas, and basal cell and clitoral gland tumors of the rat. This implies that the activated *ras* was present in the cell that clonally expanded to these benign tumors. In addition, it was recently shown that mouse epidermal cells injected

in vivo with the viral Ha-*ras* gene can be promoted with 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) to papillomas (*22*). Thus, activation of the *ras* proto-oncogene may be the initiation event in some model systems. Moreover, dormant initiated cells with the activated *ras* gene can survive surrounded by normal cells until stimulated to proliferate by some endogenous or exogenous agent.

Implications for Risk Analysis

The number of proto-oncogenes that must be activated in order to convert a normal cell into one that is tumorigenic is unknown at present. However, there is increasing evidence that the transformation of a normal cell into a tumorigenic cell involves the activation and concerted expression of several proto-oncogenes as well as, perhaps, the inactivation of suppressor genes. Continued research on mechanisms of oncogene activation in animal and *in vitro* models may provide new insights into several long-standing problems in chemical carcinogenesis and risk analysis of carcinogenesis data.

Oncogene analysis on tumors from long-term carcinogenesis studies that are employed to help identify potential human carcinogens can be useful in several ways. The analysis can help identify chemicals that can activate proto-oncogenes *in vivo* to cancer-causing genes. Model systems very susceptible to chemically induced tumors, such as the B6C3F1 mouse liver tumors, appear to be suited for this purpose (*23*). The classification of chemicals as initiators, promoters, complete carcinogens, etc., may become clearer as we better understand the sequential requirements for activation of oncogenes in the various animal model and cell culture systems. In particular, comparison of patterns of oncogene activation in spontaneously occurring and chemical-induced tumors should assist in determining mode(s) of action of a carcinogen. Low-dose and species-to-species extrapolation of risk from carcinogenic data may become more reliable from examination of oncogene activation and expression in animal model systems for carcinogenesis. These and similar approaches to explore the mechanisms by which chemicals induce tumors in animal model systems may remove some of the uncertainty in risk analysis of rodent carcinogenic data.

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